AFRL-OSR-VA-TR-2013-0145

OPTIMIZATION OF BIOFUEL PRODUCTION FROM TRANSGENIC MICROALGAE

Richard Sayre
Donald Danforth Plant Science Center

April 2013
Final Report

AIR FORCE RESEARCH LABORATORY
AF OFFICE OF SCIENTIFIC RESEARCH (AFOSR)
ARLINGTON, VIRGINIA 22203
AIR FORCE MATERIEL COMMAND

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
27 Feb 2013	Final Technical	20080815 to 20120630

4. TITLE AND SUBTITLE

OPTIMIZATION OF BIOFUEL PRODUCTION FROM TRANSGENIC MICROALGAE

5a. CONTRACT NUMBER

5b. GRANT NUMBER

FA9550-08-1-0451

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Richard Sayre

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Donald Danforth Plant Science Center
975 N. Warson Rd
St Louis Mo, 63132-2918

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)

AFOSR
875 N. Randolph St
Arlington, VA 22203

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

AFRL-OSR-VA-TR-2013-0145

12. DISTRIBUTION/AVAILABILITY STATEMENT

Distribution A: Approved for public release; distribution is unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

We have compared the proteomes and transcriptomes of Chlorella induced to produce oil in the presence of glucose at various time points. These results lead to the identification of several proteins when over-expressed in transgenic algae enhance photosynthesis by up to 80%. We have also completed the proteome analysis for the diatom Cyclotella when induced to accumulate oil after silicon starvation. The results of these studies are currently being analyzed. We have completed generation of Chlamydomonas transformants expressing carbonic anhydrase to increase the CO2 concentration near the active site of Rubisco so as to inhibit photorespiration. These transgenics have on average a 30% increase in photosynthetic rates. We have also show that by reducing chlorophyll b levels we can tune the light harvesting antennae size for increased photosynthetic efficiency and growth (30% increase) in algal monocultures. Finally, we have demonstrated that the biocompatible solvent, decane, rapidly induces oil accumulation in Chlorella shortening the time to produce oil with a glucose boost from 24 to 2 hours. This process also elevates oil accumulation in single cells in addition to autospores. As a result oil accumulation is increased 10X relative to glucose alone within the first 4 hours after

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			Richard Sayre
unclassified	unclassified	unclassified	SAR		19b. TELEPHONE NUMBER *(Include area code)* 314-587-1041

Investigator: Richard Sayre
Title: OPTIMIZATION OF BIOFUEL PRODUCTION FROM TRANSGENIC MICROALGAE Grant/Contract Number: FA9550-08-1-0451
Reporting Period: Final Report

Abstract: We have compared the proteomes and transcriptomes of Chlorella induced to produce oil in the presence of glucose at various time points. These results lead to the identification of several proteins when over-expressed in transgenic algae enhance photosynthesis by up to 80%. We have also completed the proteome analysis for the diatom Cyclotella when induced to accumulate oil after silicon starvation. The results of these studies are currently being analyzed. We have completed generation of Chlamydomonas transformants expressing carbonic anhydrase to increase the CO2 concentration near the active site of Rubisco so as to inhibit photorespiration. These transgenics have on average a 30% increase in photosynthetic rates. We have also show that by reducing chlorophyll b levels we can tune the light harvesting antennae size for increased photosynthetic efficiency and growth (30% increase) in algal monocultures. Finally, we have demonstrated that the biocompatible solvent, decane, rapidly induces oil accumulation in Chlorella shortening the time to produce oil with a glucose boost from 24 to 2 hours. This process also elevates oil accumulation in single cells in addition to autospores. As a result oil accumulation is increased 10X relative to glucose alone within the first 4 hours after treatment.

Chlorella proteomics: We have compared the proteomes of Chlorella induced to produce oil in the presence of glucose at various time points correlating with the accumulation of neutral lipids. This data has been complemented now by RNAseq analyses (in collaboration with the Pellegrini and Merchant labs) at the same time points used for the proteomics studies as well as under a variety of other growth conditions. These data will provide a more reliable database for annotation of the genome and identification of proteins. We have nearly completed the bioinformatic analyses and expect to have a draft publication in two months for submission. We have identified over 50 soluble proteins whose accumulation is altered during glucose-induced oil production (Figure 1).

Candidate genes for regulating oil and biomass accumulation from iTRAQ proteomic analysis

Name and fold change	6h/0h	12h/0h	24h/0h
Ca++/calmodulin dependent protein kinase II	0.30	0.25	0.27
Malate dehydrogenase (mt)	0.72	0.55	0.37
oxalate oxidase; rhodopsin-like GPCR superfamily	0.85	0.67	0.55
transcription factor and related HOX domain protein	0.72	0.59	0.52
triosephosphate isomerase	0.70	0.60	0.57
acylglycerol lipase activity	0.72	0.57	N/A
fructose-1,6-biphosphatase	1.00	1.76	N/A
acyl-coA-binding protein, ACBP	0.77	N/A	2.27
Phosphopantetheine-binding, acyl-carrier protein	1.28	N/A	1.90
Forkhead-associated protein	1.32	1.51	1.95
Calvin Cycle CP12	1.80	1.35	2.37
histone H2A	1.01	1.46	3.15
Cytochrome c	1.28	2.50	N/A

Mitochondria respiration
Photosynthesis/Calvin Cycle
Lipid metabolism

Figure 1. Proteins that change most in abundance following glucose-induced oil production.

Many of these are enzymes are involved in primary metabolism including respiration and the Calvin cycle. Two independent genes encoding proteins that undergo substantial changes in abundance during facultative oil induction have been over-expressed in transgenic algae. Both genes are cyanobacterial forms of FBPase that differ in their relative Kms by two fold. We observed that over-expression of the FBPase with the lower Km enhanced growth rates by 80%. We are now reconfirming these results after our move to LANL. We have also targeted the mitochondrial malate dehydrogenase, and the gloxosome cycle enzymes malate synthase and isocitrate lyase for over and under-expression to determine their impacts on oil accumulation based on data from the proteomics studies.

As part of our collaboration with the Hildebrand group, we have also carried out proteomic analyses of the diatom Cyclotella induced to accumulate oil resulting from silicon deficiency. That work is now in the analysis stage.

Chlorella transformation: Using vectors we developed the first year we have over-expressed an Arabidopsis DGAT gene in Chlorella. The resulting phenotype more rapidly accumulates TAG during glucose feeding but the total final oil content does not change dramatically. This accelerated (30%) oil accumulation will reduce production costs by reducing residence time in the bioreactor for feeding sugars

Optimization of light harvesting antennae size (Joint effort with the Photosynthetic Antennae Research Center - DOE-EFRC): Over 50% of the energy losses associated with photosynthesis is attributed to kinetic constraints between light harvesting and the initial charge separation processes. At high light intensities energy flux from the light harvesting antennae may be 100-fold greater than charge separation processes resulting in the dissipation of up to 75% of the captured energy as heat or fluorescence. One means to couple energy capture and charge separation flux more efficiently is to reduce the optical cross-section of the light harvesting antennae. We have show that by reducing chlorophyll b levels (Figure 2), we can tune the light harvesting antennae size for increased photosynthetic efficiency and growth in algal monocultures. It is hypothesized that the large antennae size offers a selective advantage in the wild due the ability to shade competing algal species and to harvest light at low flux densities. This work is now in review for publication in Science.

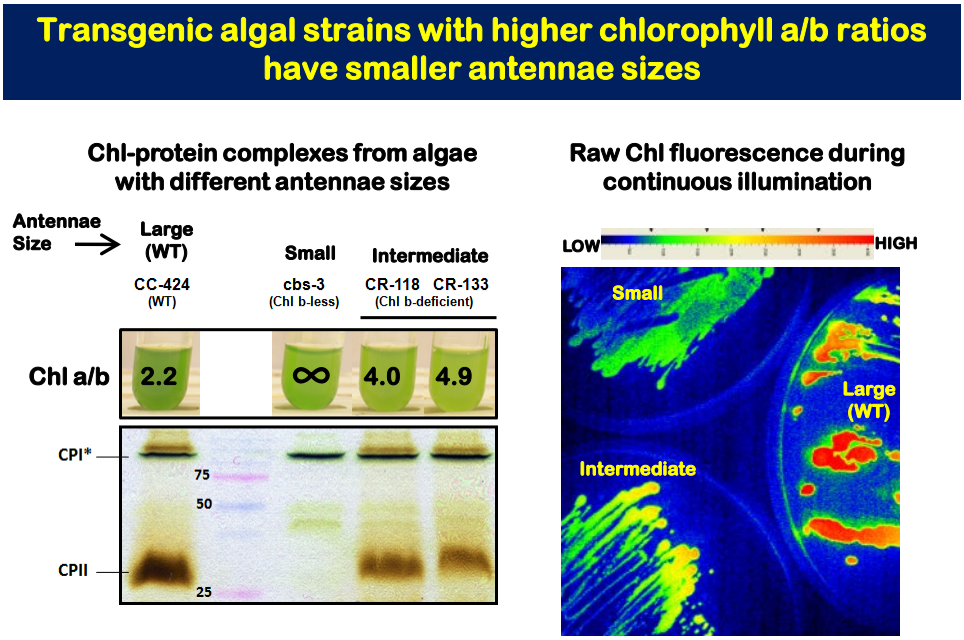


Figure 2. Relationship between chlorophyll b levels and antennae size.

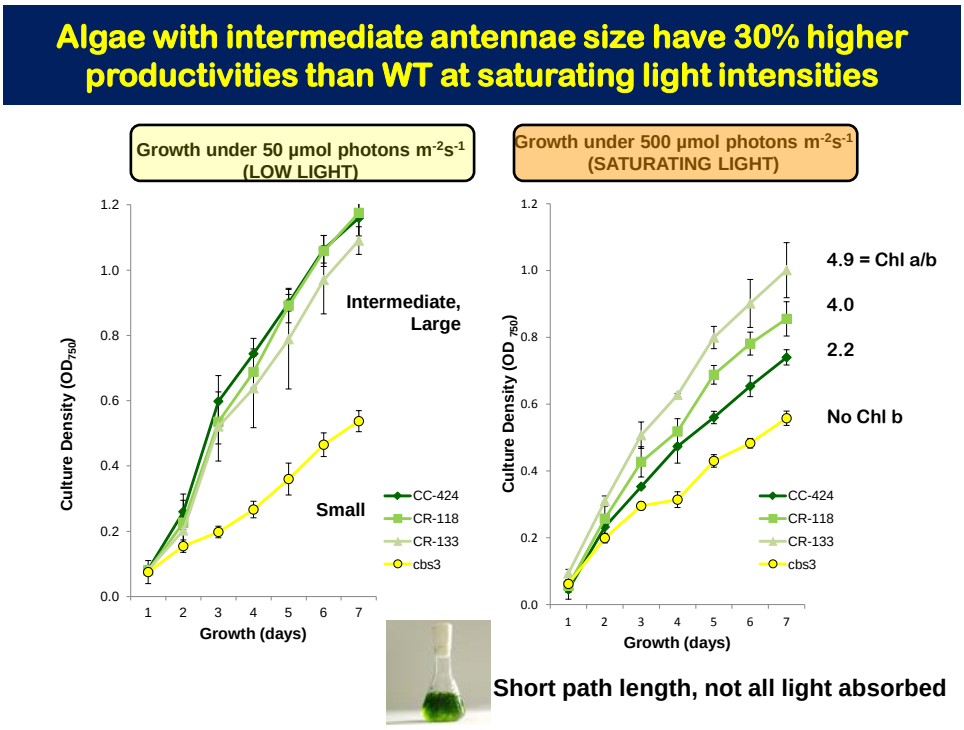


Figure 3. Enhanced growth rates at high light intensities for transgenic algae with intermediate antennae sizes (Chl a/b ratios 4 and 4.9). Chlorophyll b less mutants (small antennae) do not grow well due to an inability to carry out state transitions and cyclic photophosphorylation.

In addition, we have developed a self-adjusting antennae system that reversibly alters and optimizes the size of the light harvesting complex to maximize light use efficiency. In the figure below, we demonstrate that the antennae size (based Chl a/b ratio and chlorophyll fluorescence raise kinetics) is reversible as a function of the growth light conditions.

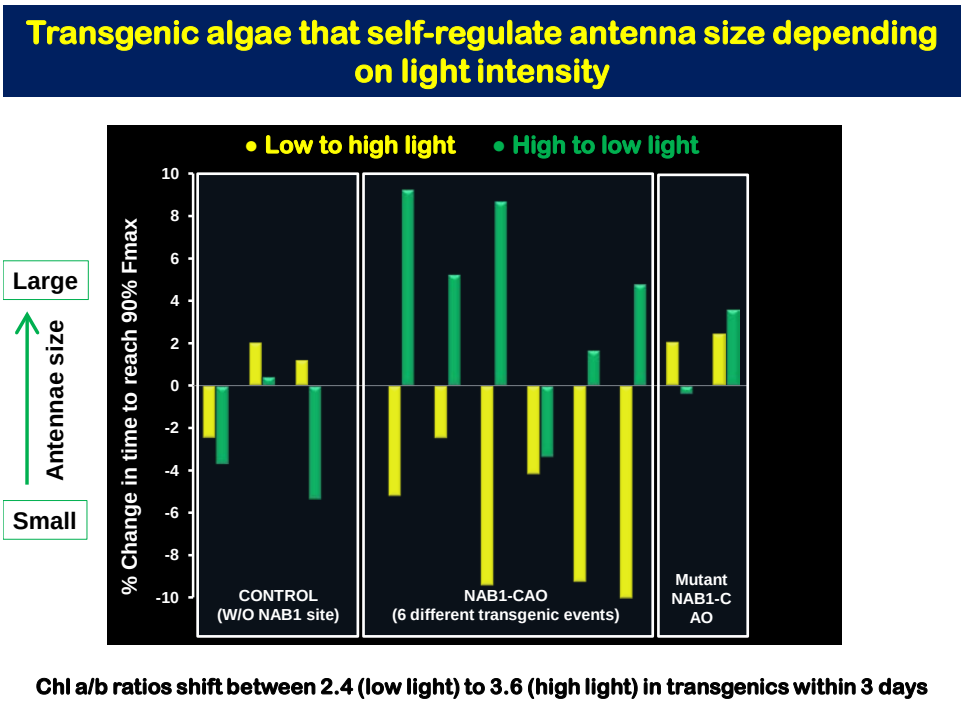
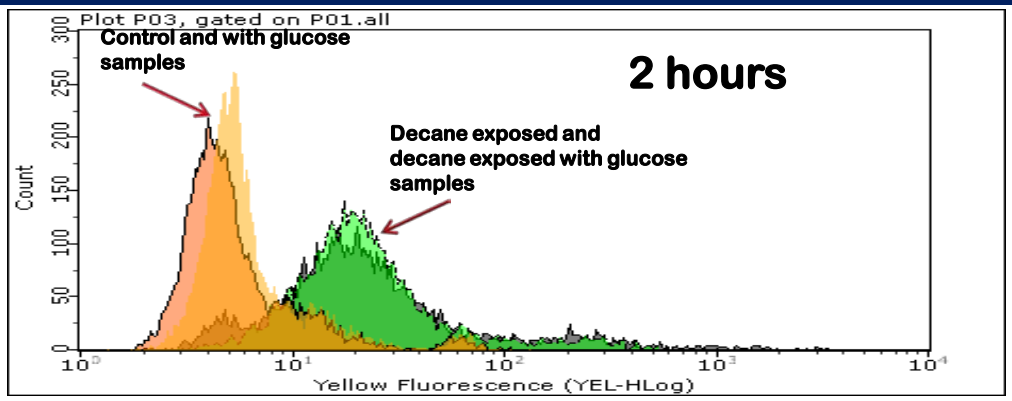
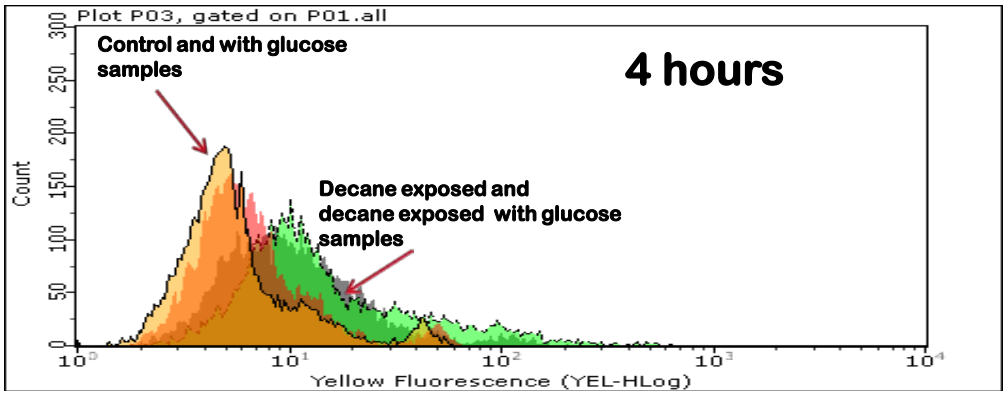


Figure 4. Reversible shift in antenna size following a three day shift in growth light intensity. The mutated NAB1 domain transgenics are unable to reversibly shift antennae size demonstrating that the NAB1 translation inhibor suppresses the activity of chlorophyll a oxygenase which is expressed under NAB1 protein control.

Non-destructive oil extraction: As part of our original investigations to develop non-destructive oil extraction technologies, we have demonstrated that the biocompatible solvent, decane, rapidly induces oil accumulation in Chlorella shortening the time to produce oil from a glucose boost from 24 to 2 hours. This process also elevates oil accumulation in single cells in addition to autospores. As a result oil accumulation is increased 10X relative to glucose alone within the first 4 hours after treatment. In the figure below are shown the results of a flow cytometry analysis of oil accumulation in algal cells treated with 0.1% decane for 2 and 4 hours. The results show a clear increase in oil accumulating cell numbers (yellow fluorescence).

Oil accumulates up to 2 hours after glucose and decane exposure





Metabolic Engineering: We are focusing on engineering both lipid synthesis and photosynthetic carbon fixation to optimize oil production. We have completed generation of Chlamydomonas transformants expressing carbonic anhydrase to increase the CO2 concentration near the active site of Rubisco so as to inhibit photorespiration. These transgenics have on average a 30% increase in photosynthetic rates. We are now linking CA to Rubisco to enhance the channeling of CO2 to Rubisco. Results indicate substantial increases in growth yield. In addition, we have completed characterization the proteomes of Chlorella induced to produce oil in the presence of glucose. We have identified over 50 soluble proteins whose accumulation is altered during glucose-induced oil production. Many of these are enzymes are involved in primary metabolism. Four independent genes encoding proteins that undergo substantial changes in abundance during facultative oil induction are targeted for altered patterns of expression in transgenic algae to determine their impact on oil production.

Hetertrophic boost of oil production: We have identified more than 50 soluble proteins whose abundance fluctuates during induction of glucose-dependent lipid accumulation in Chlorella. Many of these enzymes participate in primary carbon metabolism. A metabolic map of the proteomic response to glucose-induced lipid accumulation has been built for facultative (glucose-dependent) oil production in Chlorella. The pattern emerging from the data analysis indicates that the algae are responding to additional

reductant (glucose) by sinking electrons into oils and the reduction of sulfate to sulfide (Cys and Met). In addition, photosynthetic electron transfer is down regulated by the chloroplastic ATPase is up regulated consistent with a rebalancing of NADPH and ATP ratios in favor of ATP synthesis when grown with glucose.

Optimizing photosynthetic antennae (LHC) size for increased photosynthetic efficiency: Antisense Chl a oxygenase and chlorophyll b reductase *Chlamydomonas* transgenics were verified as having an reduced or intermediate LHC content. Photosynthetic rates at high CO2 versus air saturation levels are higher at all light intensities in the intermediate LHC transformants relative to wild-type and LHS minus mutants. Green house competition experiments with different pond depths demonstrate that has light intensities increase it is advantageous to have smaller antennae and deeper ponds to efficiently convert photons into biomass. This is the first time field trials have been conducted in the open ponds comparing biomass productivity of algae with different antennae sizes.

Reducing photorespiration: We have successfully expressed human carbonic anhydrase II (CA2) in the chloroplast genome of Chlamydomonas. CA2 catalyzes the reversible conversion of bicarbonate into CO2. The human CA2 has an exceptionally high Kcat of 10^6 s^{-1}. On plates the transgenic algae expressing CA2 have a substantially more robust growth rate than wild-type algae. In liquid culture the growth rate differences are reduced possibly associated with differential availability of CO2. The next phase is to physically link CA2 to Rubsico to reduce the CO2 diffusion distance and inhibit photorespiration.

Building a molecular tool box for Chlorella for enhanced photosynthetic growth: The promoter and terminator of the actin and ubiquitin gene have been cloned from *Chlorella* and vectors have been constructed to drive the expression of transgenes hypothesized to increase photosynthetic carbon fixation and oil yields. Techniques for monitoring lipid accumulation during glucose-dependent induction by TLC have also been standardized for algae

Conclusion: This effort was the result of a 4 year grant which was the result of a previous 1 year grant. The total time resulted in a 60 month effort. The PI moved from the Danforth Plant Center in St Louis to Department of Energy in Los Alamos New Mexico.

Publications:

- Perrine Z, Negi S and Sayre, RT (2011) Optimizing the efficiency of photosynthetic light capture in microalgae. *Science, in review*.
- Service R (2011) Algae's second try. *Science* 333:1238-1239
- Blankenship RE, Tiede DM, Barber J, Brudvig GW, Fleming G, Ghirardi M, Gunner MR, Junge W, Kramer DM, Melis A, Moore TA, Moser CC, Nocera DG, Nozik AJ, Ort DR, Parson WW, Prince RC and Sayre RT (2011**)** What is the solar energy conversion

efficiency of natural photosynthesis compared to photovoltaic cells? *Science* 332:805-809.

- Perrine Z and Sayre RT (2011) Modulating the Redox Potential of the Stable Electron Acceptor, Q_B, in Mutagenized Photosystem II Reaction Centers. *Biochemistry* 50:1454-1464.

Honors:

- Elected Fellow, Biology Division, American Association for the Advancement of Sciences (2011)